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Supplemental Information

Endogenous WNT Signaling Regulates hPSC-Derived Neural Progenitor Cell Heterogeneity and Specifies Their Regional Identity

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cells and culture conditions. All media components were from Life Technologies unless otherwise noted. For hPSC culture, the following media were used: mouse embryonic fibroblast (MEF) (1X high glucose DMEM, 10% fetal bovine serum, 1% (v/v) L-glutamine penicillin/streptomycin). H9/HES3/RiPSC hPSCs (1X DMEM-F12, 20% (v/v) Knockout Serum Replacement, 1% (v/v) non-essential amino acids, 0.5% (v/v) glutamine, 120 μM 2-mercaptoethanol [Sigma]). HUES9 (1 x Knockout DMEM, 10% (v/v) Knockout Serum Replacement, 10% (v/v) human plasmanate (Chapin Healthcare, Anaheim CA, USA) 1% (v/v) non-essential amino acids, 0.5% (v/v) glutamine, 55 μM 2-mercaptoethanol [Sigma]); All hPSC lines were maintained on feeder layers of mitotically inactivated MEFs ($2x10^4/cm^2$; Millipore). All hPSC cultures were supplemented with 30 ng/ml FGF2 (Life Technologies). MEF-CM was produced by culturing hPSC medium on MEFs for 24 hr followed by sterile filtering. Cells were routinely passaged with Accutase (Millipore), washed, and replated at a density $4.25 \times 10^4/cm^2$.

Generation of Wnt reporter hESCs. The lentiviral construct that was used to generate the WNT reporter line contained a 7xTCF-eGFP construct and puromycin resistance gene[25]. High titer lentivirus was produced as previously described[97, 98]. HUES9 hESCs were infected overnight with lentivirus. Infected pools were selected with puromycin (0.5 μg/ml) for 2 weeks. For generation of clonal hESC lines, transduced pools were then treated with 200 ng/ml purified mouse WNT3a[99] for 48 hours. Cells were dissociated with Accutase for 5 min at 37°C, triturated, and passed through a 40 μm cell strainer. Cells were then washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and resuspended at a maximum concentration of 5×10^6 cells per 100 ul. Single TOP-GFP+ cells were sorted into a Matrigel (BD)-coated 96 well plated with MEF-CM supplemented with 5 μM ROCK inhibitor Y27632 (Stemgent) and 30 ng/ml FGF2. After expansion, a total of 45 clones were screened for: (1) robust TOP-GFP expression upon WNT3a stimulation and (2) normal euploid karyotype.

Neural progenitor cell (NPC) generation, expansion, and differentiation. To initiate neural differentiation, hPSCs were cultured on Matrigel (BD Biosciences) in MEF-CM supplemented with 30 ng/ml FGF2 or TeSR™2 (Stem Cell Technologies). Cells were then detached with treatment with Accutase (Millipore) for 5 min and resuspended in neural induction media (1% N2/1% B27 without vitamin A/DMEM:F12) supplemented with 5 μM Y-267632 (Stemgent), 50 ng/ml recombinant mouse Noggin (R&D Systems), 0.5 μM Dorsomorphin (Tocris Bioscience)]. Next, 7.5×10^5 cells were pipetted to each well of a 6-well ultra low attachment plates (Corning). The plates were then placed on an orbital shaker set at 95 rpm in a 37°C/5% CO2 tissue culture incubator. The next day, the cells formed spherical clusters (embryoid bodies [EBs]) and the media was changed to neural induction media with 50 ng/ml recombinant mouse Noggin and 0.5 μM Dorsomorphin. The media was subsequently changed every other day. After 5 days in suspension culture, the EBs were then transferred to a 10 cm dish coated (3 x 6 wells per 10 cm dish) with growth factor reduced Matrigel (1:25 in KnockOut DMEM; BD Biosciences) for attachment. The plated EBs were cultured in neural induction media with 50 ng/ml recombinant mouse Noggin and 0.5 μM Dorsomorphin for an additional 7 days. Neural rosettes were cut out by dissection under an EVOS (Life Technologies) microscope. Dissected rosettes were incubated in Accutase for 5 min and then triturated to single cells with a 1 mL pipet. Rosettes were then plated onto poly-L-ornithine (PLO; 10 μg/mL; Sigma) and mouse laminin (Ln; 5 μg/mL; Sigma) coated dishes at a density of 12,500 cells/cm^2 in neural induction media supplemented with 10 ng/mL mouse FGF2 and 10 ng/ml mouse EGF2 (R&D Systems). IWP2 (Stemgent) and CHIR 98014 (CHIR; Axon Medchem) were added 2 days after EB formation. For routine maintenance, NPCs were passaged onto PLO/Ln coated plates at a density of 10,000 cells/cm^2 in neural induction media supplemented with 10 ng/mL mouse FGF2 and 10 ng/ml mouse EGF2. TOP-GFP sorted as well as IWP2- and CHIR-treated NPCs were derived and

maintained in the absence of FGF2 and EGF2. For neuronal differentiation, NPCs were dissociated with Accutase for 5 min at 37°C, triturated, and plated onto PLO/Ln coated plates at a density of 100,000 cells/cm^2. Cells were cultured in neuronal differentiation media (0.5% N2/0.5% B27 without vitamin A/DMEM:F12) supplemented with 20 ng/ml BDNF (R&D Systems), 20 ng/ml GDNF (R&D Systems), 1 µM DAPT (Tocris Bioscience), and 0.5 mM , dibutyrl-cAMP (db-cAMP; Sigma) for 4 weeks.

Quantitative PCR (Q-PCR). RNA was isolated from cells using TRIzol (Life Technologies), and treated with DNase I (Life Technologies) to remove traces of genomic DNA. Reverse transcription was performed with qScript cDNA Supermix (Quanta Biosciences). Quantitative PCR was carried out using TaqMan probes (Life Technologies) and TaqMan Fast Universal PCR Master Mix (Life Technologies) on a 7900HT Real Time PCR machine (Life Technologies), with a 10 min gradient to 95°C followed by 40 cycles at 95°C for 15s and 60°C for 1 min. Taqman gene expression assay primers (Life Technologies; **Table S4**) were used. Gene expression was normalized to 18S rRNA levels. Delta Ct values were calculated as $C_t^{target} - C_t^{18s}$. All experiments were performed with three technical replicates. Relative fold changes in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method[100]. Data are presented as the average of the biological replicates ± standard error of the mean (S.E.M).

Immunofluorescence. Cultures were gently washed twice with staining buffer (PBS w/ 1% (w/v) BSA) prior to fixation. Cultures were then fixed for 15 min at room temperature (RT) with fresh paraformaldeyde (4% (w/v)). The cultures were washed twice with staining buffer and permeabilized with 0.2% (v/v) Triton-X-100 in stain buffer for 20 min at 4°C. Cultures were then washed twice with staining buffer. Primary antibodies were incubated overnight at 4°C and then washed twice with stain buffer at RT. Secondary antibodies were incubated at RT for 1 hr. Antibodies used are listed in **Table S5**. Nucleic acids were stained for DNA with Hoechst 33342 (2 µg/ml; Life Technologies) for 5 min at room temperature. Imaging was performed using an automated confocal microscope (Olympus Fluoview 1000 with motorized stage). Quantification of images was performed by counting a minimum of 9 fields at 20x magnification. Image quantification of the data is presented as the average of these fields ± standard deviation (S.D.).

Flow cytometry and cell replating. Cells were dissociated with Accutase for 5 min at 37°C, triturated, and passed through a 40 µm cell strainer. Cells were then washed twice with FACS buffer (PBS, 10 mM EDTA, and 2% FBS) and resuspended at a maximum concentration of 5 x 10^6 cells per 100 ul. One test volume of antibody was added for each 100 µl cell suspension (**Table S5**). Cells were stained for 30 min on ice, washed, and resuspended in stain buffer. Cells were analyzed and sorted with a FACSCanto or FACSAria (BD Biosciences). Flow cytometry data was analyzed with FACSDiva software (BD Biosciences). Isotype negative controls are listed in **Table S4**. For sorting experiments in which cells were separated on the basis of GFP expression, wild-type (WT) non-fluorescing cells were used as a negative control. For replating experiments, cells were stained with appropriate antibodies and sorted into FACS buffer with 5µM Y27632 (Stemgent). Sorted cells were replated at the appropriate density and media with 10 µM Y27632.

High throughput RNA sequencing (RNA-seq). Total RNA from FACS sorted TOP-GFP$^+$ and TOP-GFP$^-$ NPCs were isolated, depleted of genomic DNA and rRNA and fragmented to ~200 bp by RNase III. After ligating the Adaptor Mix, fragmented RNA was converted to the first strand cDNA by ArrayScript Reverse Transcriptase (Ambion), size selected (100-200bp) by gel electrophoresis, and amplified by PCR using adaptor-specific primers. Deep sequencing was performed on an Illumina Genome Analyzer II. Analysis of genome-wide expression data was performed as previously described[101, 102]. Briefly, raw reads from two biologically independent samples were aligned to the reference human genome (hg19) using

TopHat. Cufflinks was used to assemble individual transcripts from the mapped reads. Cuffmerge was used to merge the assembled transcripts from the two biologically independent samples. Cuffdiff was used to calculate gene expression levels and test for the statistical significance of differences in gene expression. Reads per kilobase per million mapped reads (RPKM) were calculated for each gene and used as an estimate of expression levels.

LEGENDS FOR SUPPLEMENTAL FIGURES

Supplemental Figure S1, related to Figure 1. Generation of clonal WNT reporter hESC lines. (a) Schematic of TOP-GFP lentiviral construct. (b) Flow cytometry analysis of expanded clones after 48 hour treatment with 15 nM purified mouse WNT3a. Clone 19 (hTOP-19) displayed the highest expression of GFP after WNT3a treatment. (c) Karyotype analysis of hTOP-19. Chromosome spread indicated a normal euploid female karyotype (46XX). (d) Flow cytometry analysis of hTOP-19 hESCs treated with various concentrations of WNT3a. (e) Flow cytometry analysis of hTOP-19 hESCs treated with GSK3β inhibitor BIO. (f) Flow cytometry analysis of GFP expression in reporter expressing NPCs after 48 hours of treatment with 15 nM WNT3a or 1000 nM IWP2. Abbreviations: NFC=Non-fluorescing channel.

Supplemental Figure S2, related to Figure 1. Differentiation of hPSCs to neural progenitor cells (NPCs) and neurons. (a) Overview of differentiation protocol for differentiation of hPSCs to NPCs and neurons. The soluble factors, substrate, and culture media at each stage are shown. (b) Immunofluorescence of OCT4, NANOG, SOX2, and SOX1 in hESCs and NPCs (scale bar = 100 µm). (c) Flow cytometry analysis of SOX1 and SOX2 expression in hESCs and NPCs. Isotype controls used are listed in **Table S2** (d) Phase contrast images of hESCs and NPCs (scale bar = 100 µm). (e) Gene expression analysis of anterior/posterior (A/P) neural tube related genes in hESCs and NPCs (mean ± S.E.M, n=3 independent experiments). Populations were compared using Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Flow cytometry analysis of (f) FORSE-1 and (g) PAX6 and (m) in NPCs. Isotype controls used are listed in **Table S5**. (h) Immunofluorescence of HOXB4 in NPCs (scale bar = 200 µm). (i) Immunofluorescence of B3T in neuronal cultures (scale bar = 1 mm). (j) Immunofluorescence of MAP2 and GFAP in neuronal cultures (scale bar = 200 µm). (k) B3T and γ-Aminobutyric acid (GABA) in neurons differentiated from hESCs (scale bar = 200 µm). Abbreviations: NFC=Non-fluorescing channel.

Supplemental Figure S3, related to Figure 2. **Analysis of TOP-GFP expressing NPC populations.** (a) Gene expression analysis of *SOX2* and *NESTIN* (mean ± S.E.M, n=3 independent experiments). (b) Immunofluorescence of NESTIN, SOX1, and SOX2. (c) Reporter expressing NPCs were separated by fluorescence-based cell sorting into GFP^{HIGH} and GFP^{LOW} populations on the basis of GFP expression. GFP^{HIGH} and GFP^{LOW} NPC populations were subsequently cultured for 10 passages (>50 days) and examine for expression of WNT and A/P related genes. (d) Gene expression of WNT target gene *AXIN2* in GFP sorted NPC populations after 5 and 10 passages (mean ± S.E.M, n=3 independent experiments). (e) Gene expression analysis of A/P related genes in GFP sorted NPC populations after 5 and 10 passages (mean ± S.E.M, n=3 independent experiments). Populations were compared using Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Abbreviations: N.S. = Not statistically significant; L = GFP^{LOW}; M = GFP^{MID}, H = GFP^{HIGH}.

Supplemental Figure S4, related to Figure 3. Analysis of neurons generated from TOP-GFP expressing NPC populations. (a) Phase contrast images of neurons derived from sorted GFP expressing NPC populations (scale bar = 500 µm). (b) Immunofluorescence of mature neuronal markers MAP2 and B3T in neuronal cultures differentiated from sorted GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} NPC populations (scale bar = 200 µm). (c) Gene expression analysis of MAP2 and B3T in neuronal cultures differentiated from sorted GFP^{LOW}, GFP^{MID}, and GFP^{HIGH} NPC populations (mean ± S.E.M, n=4 independent experiments). Populations were compared to using Student's t-test. Abbreviations: N.S. = Not statistically significant

Supplemental Figure S5, related to Figure 4. Analysis of CHIR-, WNT-, IWP2-, and un-treated embryoid bodies, NPCs, and neurons. (a) Phase contrast images and size distribution of embryoid bodies (EBs) generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions (scale bar = 200 µm). (b) Size

distribution of EBs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions. The diameter of 200 EBs was measured for each condition. (c) Gene expression (mean ± S.E.M, n=3 independent experiments), (d) immunofluorescence (scale bar = 100 μm), and (e) flow cytometry analysis of NESTIN, SOX1, and SOX2 of NPCs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions. Isotype controls used are listed in **Table S2**. (f) Gene expression analysis of anterior/posterior (A/P) neural tube related genes in NPCs (mean ± S.E.M, n=3 independent experiments) generated in the presence of various WNT concentrations. Populations were compared using Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (g) Gene expression analysis of MAP2 and B3T in neuronal cultures differentiated from NPCs generated in 500 nM CHIR-, 1000 nM IWP2-, and un-treated conditions (mean ± S.E.M, n=4 independent experiments). Populations were compared to neurons differentiated from untreated (N) NPCs using Student's t-test. Abbreviations: N=None, C=CHIR 98014, I=IWP2, N.S. = Not statistically significant

Supplemental Figure S6, related to Figure 5. Analysis of stability of patterning of NPCs imposed by exogenous WNT manipulation. (a) Posterior-patterned NPCs (i.e. NPCs generated in the presence of 500 nM CHIR) were cultured without CHIR (C-N) or in the presence of 500 nM CHIR (C-C) or 1000 nM IWP2 (C-I) for 10 passages. (b) Anterior-patterned NPCs (i.e. NPCs generated in the presence of 1000 nM IWP2 were cultured without IWP2 (I-N) or in the presence of 500 nM CHIR (I-C) or 1000 nM IWP2 (I-I) for 10 passages. Expression of (c) *FOXG1* and (d) *HOXB4* was assessed in all conditions after 10 passages and compared to initial passage (P0) NPC cultures (mean ± S.E.M, n=3 independent experiments). Populations were compared using Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

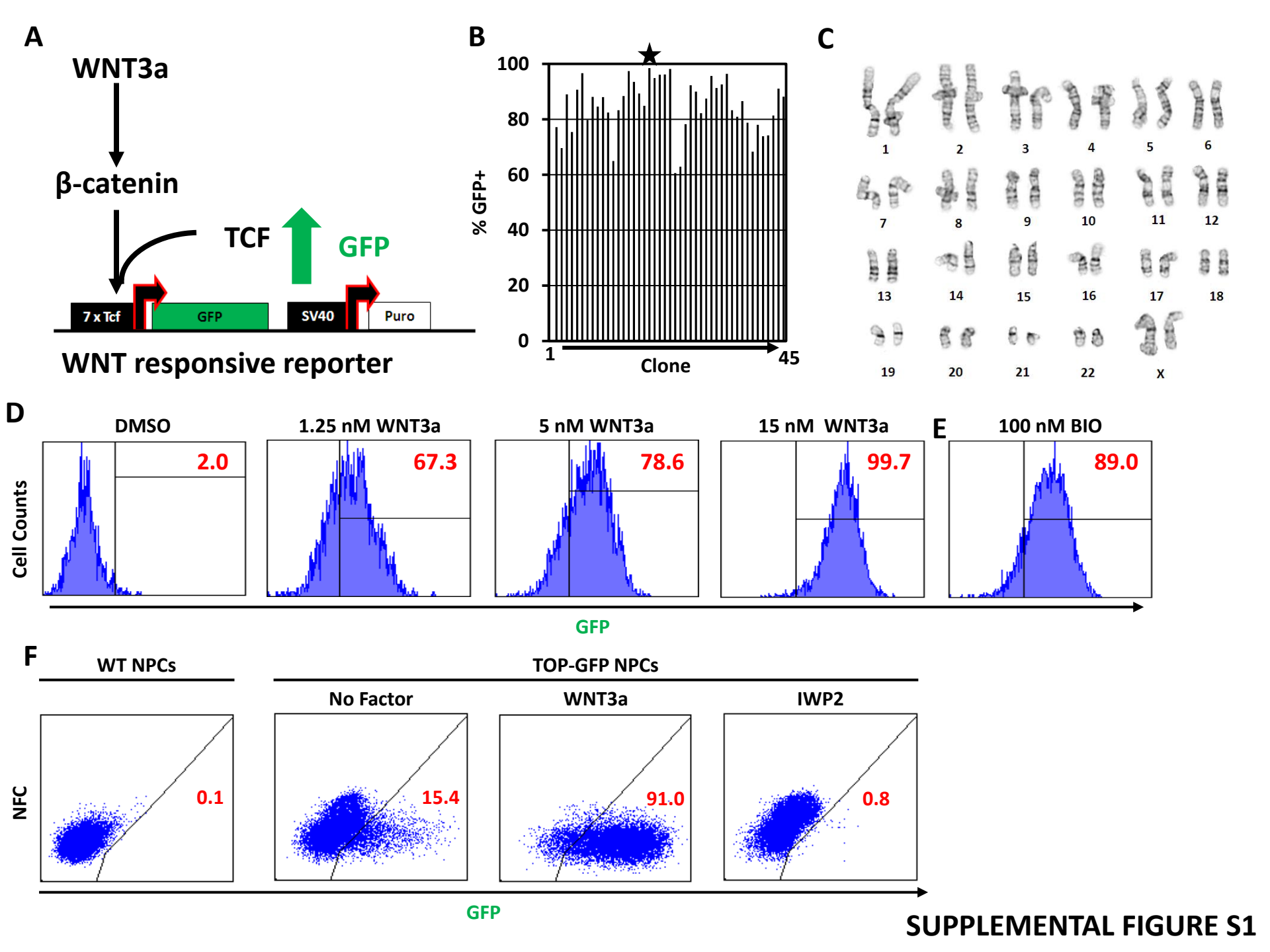
Supplemental Table S1. RNA-seq data of sorted passage 5 GFP^+ and GFP^- NPCs, related to Figure 1. RNA-seq data related to Figure 1c. Genes highlighted in light green were significantly upregulated in TOP GFP- NPCs while genes highlighted in dark green were significantly upregulated in TOP-GFP+ NPCs. Fold changes for each gene are presented as $\log_2$[(TOP-GFP+ RPKM)/(TOP-GFP- RPKM)]. The data in this table is presented in Figure 1c.

Supplemental Table S2. RNA-seq data of WNT pathway components, related to Figure 1. The data in this table is presented in Figure 1D.

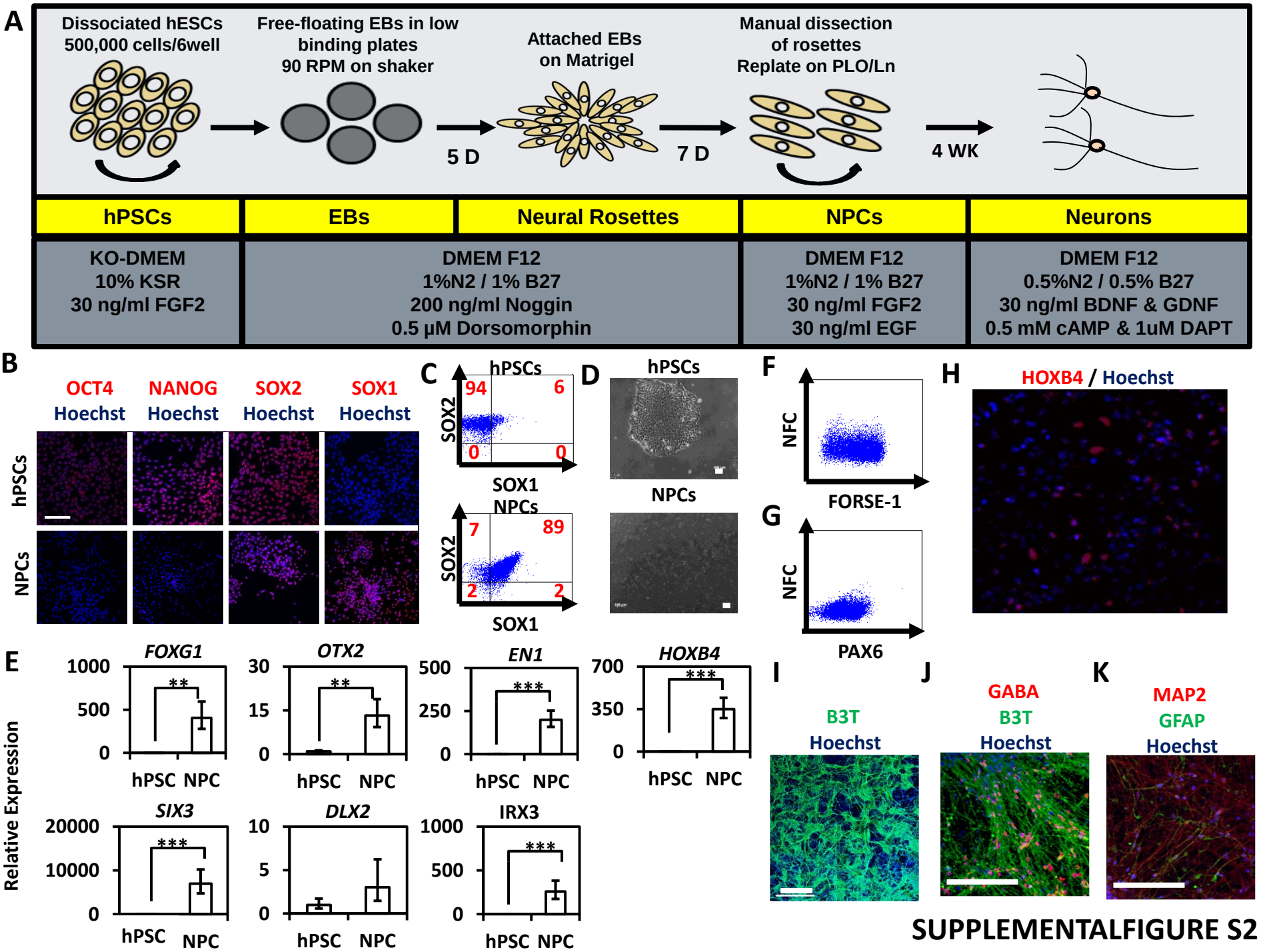
Supplemental Table S3. RNA-seq data of A/P related genes, related to Figure 1. The data in this table is presented in Figure 1E.

Supplemental Table S4. TaqMan gene expression assays used in this study.

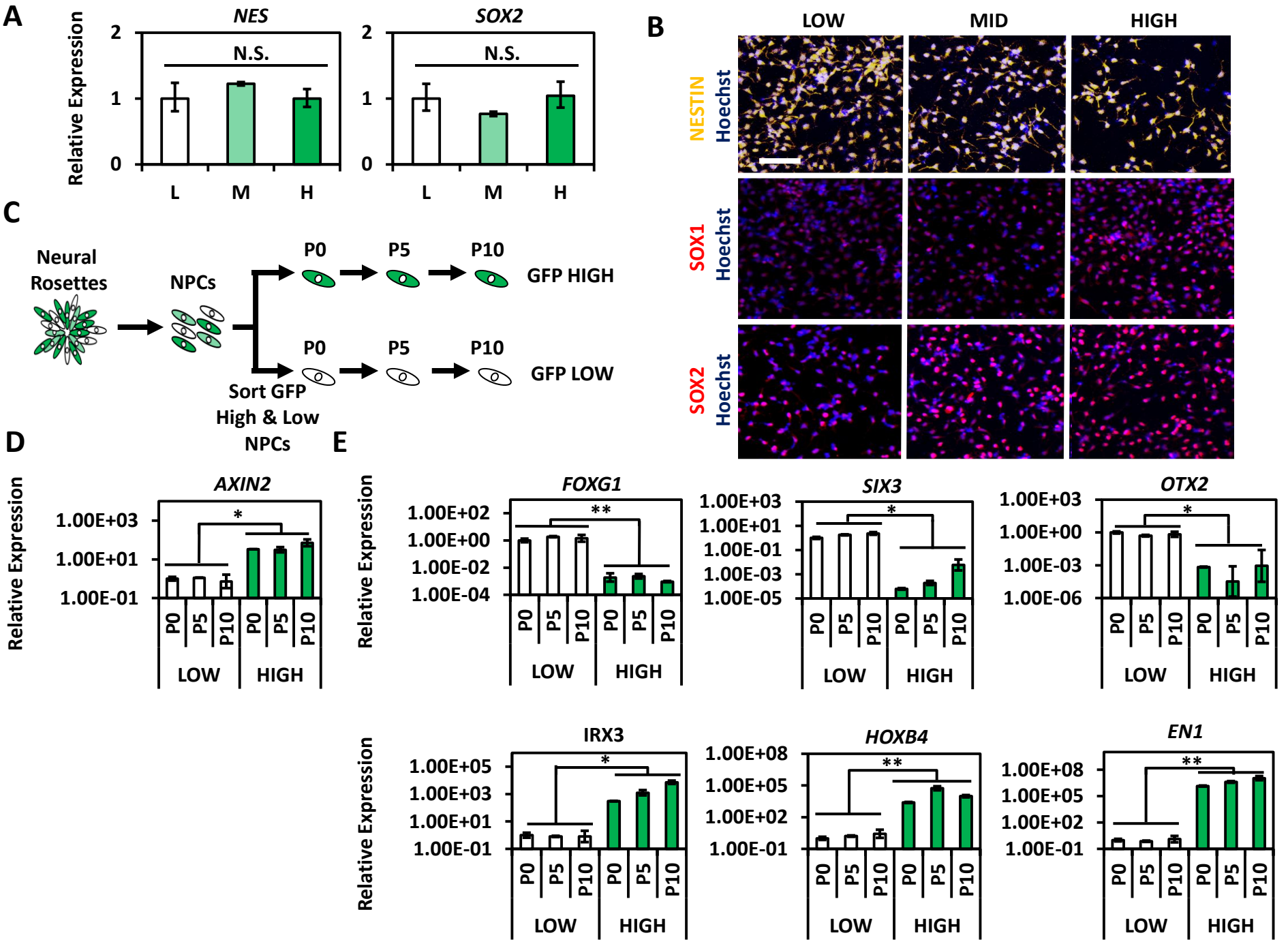
Supplemental Table S5. Antibodies used in this study.



SUPPLEMENTAL FIGURE S1



SUPPLEMENTALFIGURE S2



SUPPLEMENTAL FIGURE S3

Antibody	Vendor	Catalog #	Concentration Used
Goat anti-SOX2	Santa Cruz	SC-17320	1:50
Goat anti-OTX2	R&D Systems	AF1979	1:200
Mouse anti-B3T	Fitzgerald	10R-T136A	1:1000
Mouse anti-EN1	DSHB	4G11	1:800
Mouse anti-FORSE-1	DSHB	Concentrate	1:75
Mouse anti-GFAP	Millipore	AB360	1:500
Mouse anti-MNX1	DSHB	81.5C10	1:100
Mouse anti-Nestin	BD	560341	1:10
Mouse anti-SOX1	BD	560749	1:10
Rabbit anti-FOXG1	Abcam	AB18259	1:100
Rabbit anti-GABA	Millipore	AB15415	1:200
Rabbit anti-HOXA2	Sigma	HPA029774	1:200
Rabbit anti-HOXB4	Abcam	AB76093	1:10
Rabbit anti-LMX1A	Abcam	AB139726	1:100
Rabbit anti-MAP2	Millipore	AB5622	1:500
Rabbit anti-NANOG	Santa Cruz	SC-33759	1:50
Rabbit anti-NURR1	Millipore	AB5778	1:200
Rabbit anti-OCT4	Santa Cruz	SC-9081	1:50
Rabbit anti-TBR1	Abcam	AB31940	1:200
Alexa-647 Mouse Anti-SOX2	BD	560294	20 µl per test
PE Mouse anti-Nestin	BD	561230	5 µl per test
PE Mouse anti-PAX6	BD	561552	5 µl per test
PerCp-Cy5.5 Mouse anti-SOX1	BD	561549	5 µl per test
Alexa-647 Mouse IgG2a Isotype Control	BD	558053	20 µl per test
PE Mouse IgG1 Isotype Control	BioLegend	400113	5 ul per test
PE Mouse IgG2a Isotype Control	BD	561552	5 ul per test
PercCp-Cy5.5 MouseIgG1 Isotype Control	BD	550795	5 ul per test
Alexa 647 Donkey Anti-Goat	Life Technologies	A-21447	1:200
Alexa 647 Donkey Anti-Rabbit	Life Technologies	A-31573	1:200
Alexa 647 Donkey Anti-Mouse	Life Technologies	A-31571	1:200
Alexa 546 Donkey Anti-Goat	Life Technologies	A-11056	1:200
Alexa 546 Donkey Anti-Rabbit	Life Technologies	A-10040	1:200
Alexa 546 Donkey Anti-Mouse	Life Technologies	A-10036	1:200
Alexa 488 Donkey Anti-Goat	Life Technologies	A-11055	1:200
Alexa 488 Donkey Anti-Rabbit	Life Technologies	A-21206	1:200
Alexa 488 Donkey Anti-Mouse	Life Technologies	A-21202	1:200

SUPPLEMENTAL TABLE S5